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(54) Title: SOLID PHASE SYNTHESIS

(57) Abstract

The present invention relates to a support system for solid phase synthesis of oligomers, such as oligonucleotides, wherein the starting compound is bound to the support via a disiloxyl linkage. Furthermore, the invention relates to a method for synthesis of oligonucleotides on a solid support. The support system comprises a stable disiloxyl linkage providing high nucleoside loadings to the support and the method allows convenient non-laborious oligomer synthesis.

SOLID PHASE SYNTHESIS

Technical field

The present invention relates to a support system for solid
5 phase synthesis of oligomers, such as oligonucleotides.
Furthermore, the invention relates to a method for synthesis
of oligonucleotides on a solid support.

Background of the invention

10 Oligonucleotides are polymers built up by polycondensation
of ribonucleoside (RNA) or deoxyribonucleoside (DNA)
phosphates.

Oligonucleotides can be assembled by repetitive addition of
nucleotide monomers using solid-phase methods. Since the
15 introduction of solid-phase synthesis [R.B. Merrifield, J.
Am. Chem. Soc. 85 (1963) 2149], the following requirements
have been worked out: (1) The solid support must be
insoluble and preferably unswellable in the solvent used.
(2) Functional groups on the solid support must allow
20 covalent binding of the first nucleoside in a reproducible
manner. (3) The solid support must be chemically inert to
all reagents used during synthesis and deprotection. The
most commonly used supports are controlled pore glass beads
(CPG), silica, or polystyrene beads.
25

Below the synthesis cycle of the commonly used
phosphoramidite method is described:

1. Deprotection of the 5'-hydroxyl group in order to
30 generate the parent hydroxyl compounds. This is normally
done by treatment of the support with di- or trichloroacetic
acid in an organic solvent (for removal of protecting
groups).

2. The support is washed in order to remove traces of acid.
35 3. The 5'-hydroxyl group is reacted with the 3'-
phosphoramidite moiety of a properly protected incoming

nucleotide (A, C, G or T) in the presence of an activator
(e.g. tetrazole) to form a 3'-5'-phosphite triester.

4. Excess reagents are removed by washing with an
appropriate solvent.

5 5. Unreacted 5'-hydroxyl groups are blocked as acetates
(capping).

6. The capping reagent is removed by washing.

7. The phosphite triester is then oxidated to the
corresponding phosphate triester. This is normally done by
10 the action of aqueous iodine.

8. The oxidation reagents are removed by washing.

The process is repeated until the desired oligonucleotide
sequence has been synthesized. After synthesis, all
15 protecting groups are removed and the oligonucleotide is
cleaved from the solid support.

In the synthesis, defective oligonucleotides are produced as
a consequence of several effects, prominently premature
20 termination of synthesis, followed by capping, which results
in 5' truncated molecules, and depurination during the
synthetic cycles that is followed by strand scission during
deprotection. Recently, attention has also been directed at
the appearance of shorter, internally deleted products - so
25 called n-1 and n-2 fragments [Temsamani et al, (1995),
Nucleic Acids Research 23(11), 1841-1844]; [Fearon et al,
(1995) Nucleic acids Res., 23(14), 2754-2761].

The need for pure oligonucleotides is exemplified by the
30 requirement for high quality products in antisense therapy
[Gelfi et al, (1996), Antisense and Nucleic Acid Drug
Development, 6, 47-53], in routine diagnostics applications,
or for physicochemical and structural studies [Agback et al,
(1994) Nucleic Acids Res, 22(8), 1404-12]. Also in molecular
35 cloning impure oligonucleotides frequently reduce efficiency
and complicate interpretation of results [McClain et al,

(1986) Nucleic Acids Res. 14(16), 6770]; [Nassal, (1988)
Gene, 66(2), 279-94].

Preparative gel electrophoresis provides the best resolution
5 for purification of oligonucleotides. The method is however
laborious, often leading to considerable loss of material,
and it is poorly suited for automation and scale-up.

Chromatographic separation can solve some of these problems,
10 offering a potential for scale-up with minimal losses and
using fully automatized instruments. These positive aspects
are off-set by the rather poor resolving power of most
chromatographic systems. As a partial solution to this
problem chromatographic separation of oligonucleotides
15 labeled with affinity tags has been used. The commonly used
trityl-on oligonucleotide separation on reversed-phase
columns, or capture of 5'-thiol labelled or biotinylated
oligonucleotides on respective thiol-affinity [Bannwarth et
al, (1990), Helv. Chim. Acta, 73, 1139-1147] or avidin
20 columns [Olejnik et al, (1996), 24(2), 361-366] offer the
possibility to isolate fragments with intact 5'-ends.
However, the 5' part of depurinated molecules notoriously
contaminate oligonucleotides purified by this method.

25 A mild basic system has been proposed for partial
deprotection and cleavage of apurinic-sites with the
oligonucleotides still bound to the solid support. In this
manner the 5' ends of depurinated molecules can be discarded
before the oligonucleotides are released from the support,
30 followed by isolation of molecules with intact 5' ends [Horn
et al, (1988), Nucleic Acids Res, 16(24), 11559-71]. In
practice, this strategy was accompanied by a substantial
loss of products, due to inadvertent release of
oligonucleotides during cleavage of depurinated sites.
35

In WO92/09615 there is described the use of an alkoxysilyl
group as a linker of the oligonucleotide to the support.

This linker is inert during the synthetic cycles and it resists conditions that cleave apurinic sites. The linker is finally cleaved from the solid support with tetra butyl ammonium fluoride (TBAF) to obtain, after reversed-phase
5 separation of DMTr-containing material, an oligonucleotide with both 3'- and 5'- ends intact. However, synthesis of this support was laborious and inconvenient. Due to low reactivity of the functional group of the linker the degree of substitution of the support becomes low which leads to
10 insufficient nucleoside loadings of the support. Thus, this method is not suitable for preparation of support useful for large scale synthesis.

Summary of the invention
15 According to a first aspect, the invention provides a support system for solid phase synthesis of oligomers. The support system comprises a support, a linker and a starting compound of the oligomer. The starting compound is bound to the support via a disiloxyl linkage. The disiloxyl function
20 is linked to a hydroxyl group on the support. The functional groups connected to the disiloxyl group are very reactive allowing for reproducible and controlled loading of the starting compounds.

25 The support system of the invention is easier to produce compared to prior art systems and provides for high loadings to the support. According to the invention high loading values are obtained for the starting nucleoside. These loadings, often higher than 200 μmol/g, are required for
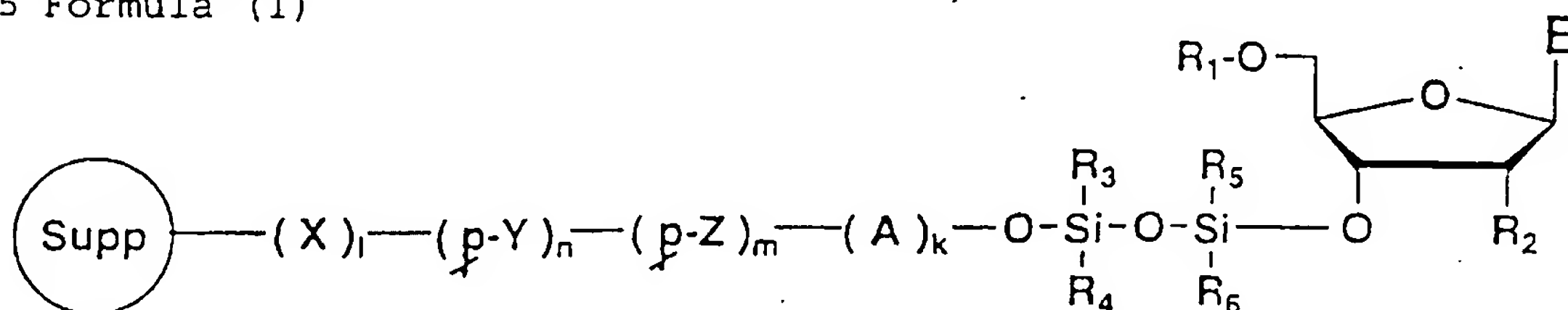30 cost-effective large scale synthesis.

The linkage is inert during the synthesis cycles and resists conditions that cleave apurinic sites.

35 In a preferred embodiment, the starting compound is a nucleoside and the solid phase synthesis is used for the synthesis of oligonucleotides.

Supports with immobilized oligonucleotides can be used as
hybridization affinity matrices. Some possible applications
of such supports are: purification of DNA-binding proteins,
5 affinity purification of plasmids, as a support for gene
assembly (from oligonucleotides) and for diagnostic
purposes, etc.

In the new support system of the present invention the first
10 nucleoside is bound to the support via a disiloxyl linkage
and the system is preferably represented by the following
formula (I).

15 Formula (I)

wherein

B is a ribonucleoside or deoxyribonucleoside base; R_2 is -H,
-OH, or OR_7 in which R_7 is a protecting group; R_1 is a
25 protecting group; R_3, R_4, R_5, R_6 taken separately each
represent alkyl, aryl, cycloalkyl, alkenyl, aralkyl,
cycloalkylalkyl, alkyloxy, aryloxy, cycloalkyloxy,
alkenyloxy and aralkyloxy; Supp is a solid support; X is an
anchoring group used for covalent bonding to the support;
30 $(p-Y)_n$ and $(p-Z)_m$ are oligophosphotriester linkers, wherein
p represents a phosphotriester, Y and Z are independently
selected from a nucleoside and a rest of a diol, A is an
alifatic or aromatic group, n is a number between 0-50,
preferably 0-10, and k, l, m are each a number of 0 or 1,
35 with the proviso that when m and n are 0 then l and k are 0
and with the proviso that when m = 1 then k is 0 and X is O
or S.

The protecting groups R_1 and R_7 are protecting groups
usually used for protection of 5´and 2´ position of ribo-
and deoxiribonucleosides. R_1 may bee selected from a trityl,
5 monomethoxy trityl, dimethoxytrityl, pixyl or other higher
alkoxy-substituted trityl- protecting groups.
R_7 may bee selected from tertbutyldimehylsilyl (TBDMS),
methoxytetrahydropyranoyl (MTHP), tetrahydropyranoyl, methyl
or allyl.
10

In a preferred embodiment of the invention R_3, R_4, R_5, R_6
are isopropyl. The choice of R_3, R_4, R_5, R_6 is dictated by
stability vs. lability requirements of the disiloxyl linker.
It is known that these properties can easily be controlled
15 by modifying electron donating parameters of the
substituents.

X can be any anchoring group, preferably O, S or an amide
function, provided it is stable to the conditions used under
20 synthesis and the reagent to cleave apurinic sites. The same
proviso applies also to the $(p-Y)_n$ linker and $(p-Z)_m$. Thus,
there are no other restrictions on Y and Z.

Z is exemplified by a tetraethylene glycol residue.
25

A wide range of porous as well as non-porous solid supports
can be used as supports in methods according to the present
invention. The group of preferred supports includes cross
linked polystyrenes, silica, polysaccharides, crosslinked
30 polysaccharides and various glasses.

When the oligophosphotriester linker $(p-Y)_n$ is present in
the above formula a support is provided resulting in even
greater oligonucleotide purity than without said linker. By
35 preparing the supports through a series of synthetic cycles
before addition of the cleavable disiloxyl linker and
synthesis of the desired oligonucleotide, any nonspecific

sites of synthesis will be neutralized. This linker gives improved contact between the starting nucleoside and an incoming reagent and ensures that also oligonucleotides starting from sites not intended for synthesis on the
5 support none the less contribute to the production of the desired oligonucleotide.

According to a second aspect the invention provides a method for oligonucleotide synthesis on a solid support Supp. The
10 method comprises the steps:

(i) preparing a support system as defined above;

(ii) condensation of nucleotides onto the first nucleoside of the support system to synthesize an oligonucleotide;

(iii) removal of all protecting groups on the
15 oligonucleotide exept the 5'-protecting group, and cleavage of apurinic sites formed during acid-catalysed deprotection;

(iv) cleavage of the full length product from the support; and

(v) purification of the oligonucleotide.
20

In step (i) an oligophosphotriester linker $(p-Y)_n$ is synthesized on the solid support and a starting nucleoside is bound to the linker via a $(p-z)_m$ linker and a disiloxyl group.
25

According to one embodiment of the method, the disiloxyl linkage is cleaved selectively according to known methods (Markiewicz W.T., 1979, Journal of Chemical Reserche (M) 0181-0197) with a compound containing fluoride ions, before
30 step (v). A preferred fluoride containing compound is tetra alkyl ammonium fluoride. Trityl ammonium hydrogene fluoride is also suitable. The purification is performed by reversed phase chromatography, using the 5'-protecting group as an affinity handle.
35

According to an alternative embodiment of the method according to the invention, step (v) is performed by

exonuclease treatment whereby non-protected oligonucleotides
will be digested. This embodiment is especially suitable for
in situ synthesis where chromatography is not possible.

5 **Detailed description of the invention**
The invention will be described more closely below in
association with an Experimental part and the accompanying
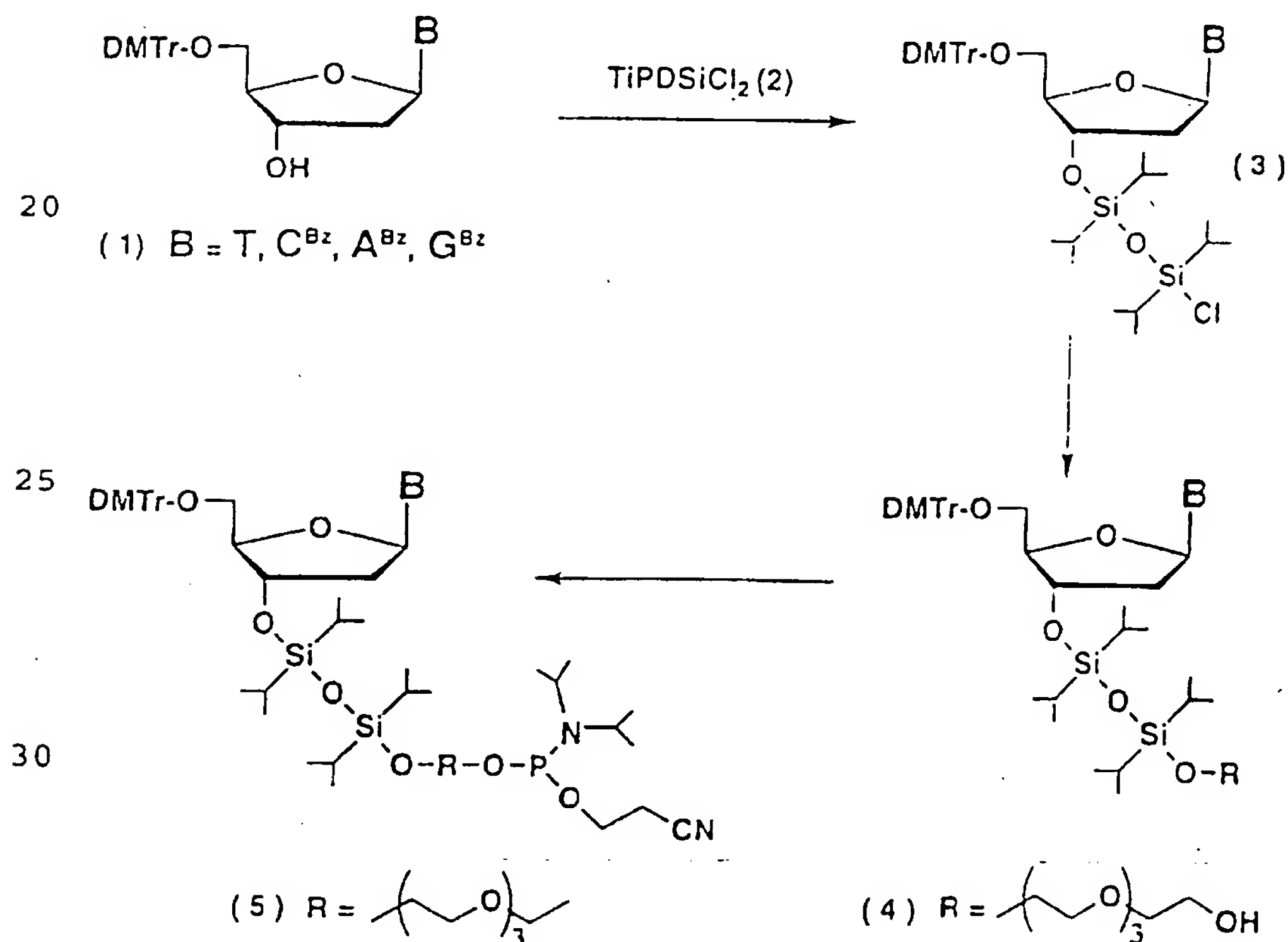drawings, in which

10 **Figure 1** shows starting points for oligonucleotide
synthesis with or without an oligonucleotide linker (**14**
and **13**, respectively) and the support after accomplished
synthesis (**15**).

15 **Figure 2** shows the results of synthesis of a purine rich
$(GA)_{40}$ oligonucleotide. A) A reverse phase HPLC
chromatogram showing the trityl containing material. The
product from a standard support appears as a much broader
peak with a large proportion of material appearing later
20 compared to the sharp and symmetrical peak from the novel
support. B) Products collected from the peaks in **A** were
5' labelled and separated on a denaturing polyacrylamide
gel. An autoradiogram showing the oligonucleotide obtained
using a standard support on the top and material
25 synthesized on the novel support below. C and D) The same
gel as in B was scanned on a Phosphorimager (Molecular
Dynamics). The data are presented as line graphs with
standard support in C and the novel support in D. The n-1
and n-2 peaks are marked in C.

30
Figure 3 shows circularization of a 91-mer padlock probe.
The term "padlock probe" means a probe which is able to
circularize on its target sequence and is described in
Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M.,
35 Chowdahry, B.P. and Landegren, U. (1994) Science, **265**
(5181), 2085-8, to which reference is made. The
oligonucleotide was labelled at the 5'-end and the two

ends were ligated using an excess of a complementary
oligonucleotide as template. The reaction was performed
for several cycles of denaturation and ligation using a
thermostable ligase. Lane 1: no ligase. Lanes 2,3 and 4:
5 1, 2 and 3 cycles of ligation, respectively. All full
length (n) oligonucleotides were circularized in the first
round of ligation.
The preparation of the support system according to the
invention can be performed according to scheme 1
10 illustrated below:

SCHEME 1

15

20 (1) B = T, C^{Bz}, A^{Bz}, G^{Bz}

(3)

25

30

(5) R = $\left(\text{O} \right)_3$ (4) R = $\left(\text{O} \right)_3$ OH

35

This procedure comprises the following steps:
1. Silylation of the 5'-dimethoxytritylated and
appropriately protected nucleosides using 1,3-dichloro-
1,1,3,3-tetraisopropyl disiloxane in dry pyridine with
5 addition of imidazol.
2. Reacting of the formed monochloroderivative (3) with an
diol e.g. tetraethylene glycol to obtain derivative (4).
3.Introduction of phosphoramidite function to the free
hydroxyl group located at the end of the 3'-linker.
10

According to the invention the homobifunctional, readily
available reagent 1,3-dichloro-1,1,3,3-tetraisopropyl
disiloxane (2)(scheme 1) was used. In other words, the
reagent used in the present invention has two reactive
15 functions which enables high and controllable
substitution.

A disiloxyl residue was applied as a stable linker
function between a nucleoside and a solid support. The
20 reactive intermediates (3) or (5) were synthesized in one
or three steps, respectively according to Scheme 1 above.

No attempts were made to isolate intermediate (3),
therefore silylation of appropriately protected
25 nucleosides (1) were performed using only a slight excess
of (2). The addition of an excess.of an 13-atoms long
chain diol (tetraethyleneglycol) resulted in the formation
of an unsymmetrical disiloxyl derivative (4), used
subsequently for the synthesis of phosphoroamidite (5).
30

The invention requires a support functionalized with
hydroxyl groups linked to the support through
nonhydrolyzable bonds. Mono R, hydroxyalkyl-derivatized
polystyrene particles (from Pharmacia) was used without
35 modifications. Derivatization of CPG proceeded according
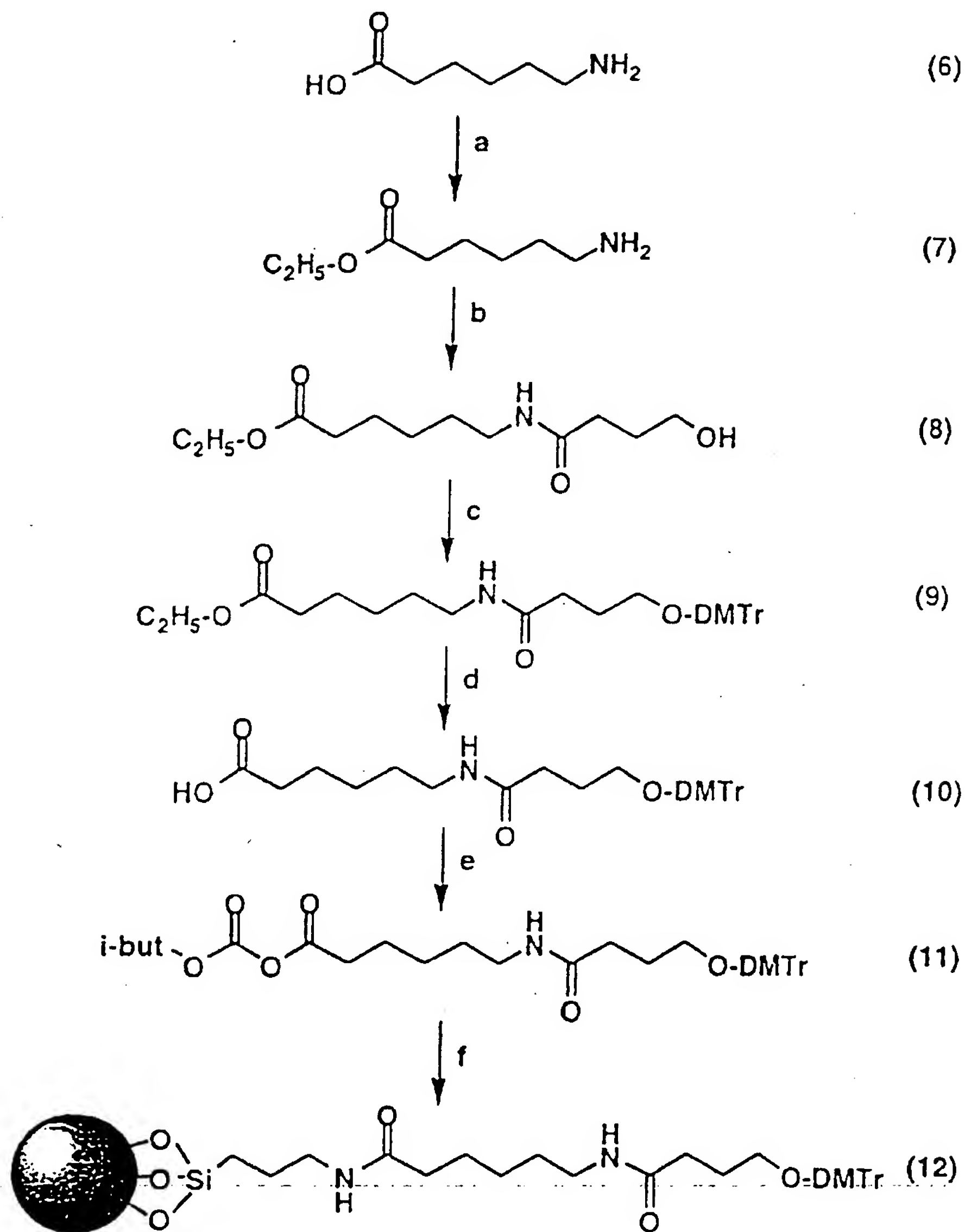to Scheme 2, below.

SCHEME 2

a) thionyl chloride, ethanol; b) γ-butyrolacton, triethylamine;
c) dimethoxyrtityl chloride, pyridine; d) NaOH, triethylamine,
e) isobutyl chloroformate; f) aminopropyl-CPG

This scheme includes the following reactions:

a) refluxing of 6-aminohexanoic acid in dry ethanol with addition of thionyl chloride to achieve esterification of carboxyl group.

5 b) acylation of the amino group with γ-butyrolactone to form compound (8).

c) protection of the introduced hydroxyl group with dimethoxytrityl group performed by DMTrCl in dry pyridine.

d) hydrolysis of the ester group with NaOH and formation of

10 the triethylammonium salt of the resulting acid.

e) activation of the carboxyl group by preparing a mixed anhydride upon addition of isobutyl chloroformate.

f) coupling of the activated reagent to the CPG support derivatized with amino functions to form support (12).

15

The CPG was activated by conventional aminopropylsilanizing [Pon, (1993) In Agraval, S. (ed.), Methods in Molecular Biology. Protocols for Oligonucleotides and Analogs. Humana Press Inc.]. Prior to

20 further derivatization, the amino CPG support was treated with trichloracetic acid in dichlormethane according to Pon [Pon, (1993), supra]. Compound (10) was prepared in high yield starting from an inexpensive 6-aminohexanoic acid and γ-butyrolacton. It was conveniently converted to

25 the mixed anhydride (11) in a reaction with isobutylchloroformate, and immediately used for coupling to CPG, see Scheme 2. Using different ratios of (11) and a solid support, loadings ranging from 15 to 60 μmol/g were obtained. The derivatized support was exhaustively capped

30 with acetic anhydride and silanized with trimethylsilylchloride, TMSCl.

EXPERIMENTAL PROCEDURES

Controlled-pore glass (CPG) (1000 Å) was obtained from CPG

35 Inc. (Fairfield, USA) and was aminopropylsilanized according to Pon et al. [Pon, 1993, supra]. Crosslinked polystyrene

particles (10 μm diameter) derivatized with hydroxylalkyl functions (Mono R) (from Pharmacia, Uppsala, Sweden).

Oligonucleotide syntheses were performed either on an ABI
5   394 DNA Synthesizer (Perkin Elmer) or on a Gene Assembler Plus (Pharmacia Biotech AB) instrument. Analytical liquid chromatography of the synthesized oligonucleotides was performed on a Hitachi-Merck La Chrom HPLC system equipped with a LiChrospher RP 18 (5 mm) column (Merck) and using a
10 linear gradient of solvent A: acetonitrile 5 % v/v in triethylammonium acetate 0.1 M, pH 7.0 and solvent B: acetonitrile 40 % v/v in triethylammonium acetate 0.1 M, pH 7.0. Preparative separations were made on an FPLC system (Pharmacia Biotech AB) using a reversed-phase Pep RPC 10/10
15 column (Pharmacia) and the above solvent gradient.

Example 1: Synthesis of nucleoside derivatives

5'-Dimethoxytrityl-3'-O-1,1,3,3-tetraisopropyl-3-
20 **tetraethylen-glycoloxy-disiloxyl thymidine (4)(B=T).**
5'-DMTr-thymidine (1) (1.30 g, 2.3 mmol)and imidazol (0.32 g, 4.8 mmol) were dried by coevaporation with dry pyridine and dissolved in 20 ml of dry pyridine. 1,3-Dichloro-1,1,3,3-tetraisopropyl disiloxane (2) (0.75 g, 2.4 mmol) was
25 added and the mixture was stirred at 20°C for 3 hrs to achieve complete consumption of starting material. Tetraethylene glycol (3.9 g, 23 mmol) was added to the formed compound (3) (see Scheme 1) and the mixture was stirred for 6 hr. Pure compound (4) was isolated as an oil
30 (1.62 g, 72%), following standard bicarbonate work-up, extraction with dichloromethane, evaporation of the organic phase, and flash column chromatography. ^{1}H-NMR (CDCl₃): 0.85-1.05 (m, 28 H), 1.43 (s, 3 H), 2.25-2.42 (m, 2 H), 2.85 (s, broad, 1 H), 3.27-3.52 (dd, 2 H), 3.54-3.76 (m, 12 H),
35 3.79 (s, 3 H), 3.80-3.85 (m, 2 H), 4.09 (m, 1 H), 4.68 (m, 1

H), 6.40 (t, 1 H), 6.83 (d, 4 H), 7.23-7.40 (m, 9 H), 7.63 (d, 1 H), 9.19 (s, broad, 1 H).

The other three nucleoside derivatives were obtained in a 5 similar manner.

Example 2: Synthesis of phosphoramidites

Synthesis of a (2-cyanoethyl) N,N-diisopropylphosphoramidite
10 **(5) (B=T).** The thymidine derivative (4) (1.50 g, 1.53 mmol) was dried by coevaporation with toluene (20 ml) and dissolved in anhydrous dichlormethane (15 ml). To this magnetically stirred solution dry triethylamine (0.85 ml, 6.0 mmol) was added followed by 2-cyanoethyl-N,N-
15 diisopropylaminophosphochloridate (710 mg, 3.0 mmol). After

15 min stirring at 20°C, TLC showed consumption of all starting material and formation of a single product. The reaction mixture was quickly partitioned between saturated aqueous sodium bicarbonate and dichloromethane and extracted
20 with dichlormethane (2x50 ml). The residue obtained after evaporation of the organic phase was dried by coevaporation with toluene and purified on a short silica gel column, prepared and eluted with CH_2Cl_2/Et3N 9/1 v/v. Fractions containing the desired product were combined, evaporated in
25 vacuo, coevaporated with dry

triethylamine, and dried in high vacuum to yield 1.57 g (87 %) of an oil; ^{31}P-NMR ($CDCl_3$ + 2 drops of triethylamine) 148.61 ppm.
30

The remaining amidites were prepared as above.

Example 3: Construction of hydroxyalkyl-derivatized CPG support (14) (See Fig. 1)
35 --

Asymmetrical anhydride (11) (see Scheme 2) was used for derivatization of aminopropylated CPG. The substitution

level was analyzed based on DMTr cation release as described
in [Gait, (ed) (1984) Oligonucleotide synthesis; a practical
approach.. IRL Press]. CPG derivatized to the extent of 28
µmol/g was selected for further experiments. Several DNA
5 synthesis columns were loaded with this support (ca 10 mg
each), and they were subjected to 10 coupling cycles with a
standard thymidine amidite, followed by coupling of one of
the nucleoside amidites (5) to obtain support (14). These
supports were used for oligonucleotide synthesis.
10

Example 4: Construction of derivatized polystyrene support
- -
Method A: Construction of support (13), Fig. 1.
Several portions of a cross-linked polystyrene support (Mono
15 R)(from Pharmacia) (0,50 g) were dried by coevaporation with
pyridine and suspended in pyridine (2 ml). Different volumes
of compound (3) (Scheme 1), prepared at a 1 mmol scale in
pyridine (10 ml), see Example 1 above, were added to the
above suspensions and the mixtures were shaken at 20°C for 2
20 hr. All activated sites that could theoretically form in the
reaction of unreacted (2) with the solid support were
quenched by the addition of methanol (5 ml). After 1 hr
shaking, the mixture was filtered, dried by washing with dry
pyridine, and transferred back to the stoppered flask, where
25 unreacted hydroxyl groups were capped with Ac_2O/DMAP (4-
dimethylaminopyridine)/pyridine for 2 hrs. The amounts of
DMTr thymidine bound directly to the support via the
disiloxyl bond (13) were checked spectrophotometrically as
above.
30

Method B: Construction of support (14), Fig. 1
A polystyrene support (10 mg), derivatized with hydroxyalkyl
groups, was packed into cassettes for synthesis of
oligonucleotides on a Gene Assembler Plus instrument
35 (Pharmacia). These packed supports were subjected to one
coupling cycle using a standard thymidine amidite that was 5

times more diluted than recommended in standard coupling procedures. The support was extensively capped (10 min) on the machine operating in a manual mode. Under the above conditions the DMTr release experiments gave values

5 comparable to those from the orginal 0.2 μmol supports. All supports were further derivatized by coupling 9 consecutive thymidine nucleotides, followed by coupling of the appropriate amidite (5) from Example 2 above. These couplings were performed using standard amidite

10 concentrations and synthesis protocols.

Example 5: Solid-phase synthesis of oligodeoxynucleotides (15), Fig. 1

----

15 **Using the ABI 394 DNA Synthesizer and CPG supports**
The CPG support (14) described above was used for oligonucleotide synthesis. All couplings were performed using amidites protected by a benzoyl group at the exocyclic amine functions, under conditions recommended by the

20 manufacturer for 0.2 μmol scale synthesis, except that nucleoside amidites were used at half the recommended concentrations. The final DMTr groups were left on the synthesized oligonucleotide.

25 **Synthesis on a Gene Assembler (Pharmacia) using the polystyrene support**

All syntheses were done at the 0.2 μmol scale using PAC amidites (Pharmacia Biotech AB), according to the manufacturer's instructions and without any changes in the

30 recommended amidite concentrations. Supports constructed according to methods A (13) or B (14) in Example 4 above were used, see also Fig. 1.

Normal scale reaction of standard nucleoside amidites or the

35 modified amidite (5) with hydroxyalkyl-polystyrene support resulted in a very high loading. Quantitative analysis of

the released DMTr-group revealed loadings of as much as 250 to 290 μmol/g. Also functionalizations involving an excess of reagent (3) gave a relatively high degree of substitution. Reaction of 0.5 mmol of (3) per 1 g of solid support at 20°C

5 for 2 hrs resulted in a support loaded at 180 μmol/g. These comparatively large numbers were a consequence of the high density of hydroxyl groups on the support and of the high reactivity of the reagents used. Such highly derivatized supports are valuble for large-scale synthesis of short

10 therapeutic oligonucleotides. For the synthesis of relatively long oligonucleotides measures had to be taken to limit this high degree of substitution. A satisfactory loading (38 μmol/g) could be obtained by using only 0.1 mmol of (3) per 1 g of support. Several-fold dilution of (5)

15 under the recommended 0.1 M concentration was the best method for direct incorporation of (5) on the polystyrene support. The same dilution method was applied to the first standard amidite used for synthesis of the polystyrene version of the support (14). All coupling reactions

20 described above were followed by an extensive capping procedure to block any unreacted hydroxyl groups.

Example 6: Deprotection and purification of the synthetic oligodeoxynucleotides

25 ---
Different deprotection procedures were used, depending on the type of support that was applied in the synthesis.

CPG-anchored oligonucleotides. A syringe filled with a

30 mixture of Et₃N/EtOH 1:1 v/v was connected to a cassette containing support for oligonucleotide synthesis. Treatment of the support with base proceeded for 3 hrs at 20°C, with the occasional addition of a new aliquot of the solvent to the cassette. The support was washed with ethanol (2 ml),

35 water (2x2 ml), dried with acetonitrile (3x2 ml), and after opening the cassette the solid support was transfered to a

Sarstedt screw-lock tube. Tetrabutyl ammonium fluoride (TBAF) 0.5 M in dry tetrahydrofuran (THF) (200 µl) was added and the mixture was incubated for 4 hr at 20°C. The cleavage of the disiloxyl linker could alternatively be done using
5 200 ml of 0.5 M TBAF in dry DMF at 65°C for 30 min. Concentrated aq. NH$_3$ (2 ml) was introduced and the mixture was placed in a 65°C oven for 12 hrs. After partial concentration the oligonucleotide was desalted on a NAP 10 Sephadex column (Pharmacia Biotech AB) and analyzed by HPLC
10 on a RP 18 column. Preparative runs were done on an FPLC using a reversed-phase Pep RPC column. Care was taken not to fractionate the hydrophobic trityl-containing product but rather to collect the whole peak, which closely resembles separations on disposable RP cartridges (Sep-Pak, C$_{18}$,
15 Waters, USA). After evaporation, the final removal of the DMTr groups was done using 80% aq. acetic acid for 20 min at 20°C, with subsequent evaporation of the acid. Alternatively, oligonucleotides phosphorylated at their 5'-position by the Tr-S phosphorylating reagent were finally deprotected
20 according to the published procedure [Connolly, (1987) Tetrahedron Lett., 28(4), 463-466].

Polystyrene support-anchored oligonucleotides. After completed synthesis, the support was transfered from the
25 cassette into a Sarstedt tube and subjected to treatment with concentrated aq. NH$_3$ for 90 min at 65°C. After cooling, the particles were briefly centrifuged and the upper liquid phase was removed. The solid-support was washed 3 times with 2 ml of water and dried by washing with acetonitrile. Two
30 hundred µl of 0.5 M TBAF in THF was added and the mixture was incubated for 4 hr. Finally, the mixture was diluted with 0.8 ml water and the oligonucleotide was desalted on a NAP 10 Sephadex column. The further deprotection and purification steps follow exactly those described for CPG-
35 bound oligonucleotides.

Example 7: Electrophoretic analysis of oligonucleotides

Oligonucleotides to be analyzed electrophoretically were
5 labelled with ^{32}P at their 5'-end using polynucleotide
kinase in a 50 μl reaction volume of 50 mM KAc, 10 mM MgCl$_2$,
10 mM Tris-HAc (pH 7.5), 10 μCi (g-^{32}P)ATP (3000 Ci/mmol),
and 10 U polynucleotide kinase (Amersham) at 37°C for 30 min.
The labelling reaction was stopped by desalting on a
10 Sephadex G-50 spin column, followed by incubation at 65°C for
5 min. All oligonucleotides analyzed electrophoretically in
this study were synthesized with a 5'-phosphate to ensure
that cleaved apurinic oligonucleotides would label with the
same efficiency as the full-length molecules. After
15 separation on a denaturing 6% polyacrylamide gel the
radioactivity was recorded by autoradiography (Amersham
Hyperfilm) or, for quantitative measurements of band
intensities, scanned on a Phosphorimager instrument
(Molecular Dynamics).

20

Example 8: Purine-rich oligonucleotides

The risk of depuration of an oligonucleotide increases
with the number of purines and the total length of the
25 oligonucleotide. An increased amount of purines in an
oligodeoxynucleotide gives a high probability of its
depurination and the following brake-down. To show the
capability of the new method to eliminate these short
sequences, two 81-mer (AG)$_{40}$T sequences were synthesized in
30 parallel using standard and novel CPG-based supports (14),
respectively. Partially deprotected 5'-DMTr-substituted
oligonucleotides were analyzed and isolated by HPLC. Both
products were detritylated, 5'-^{32}P kinased and
electrophoretically separated on a denaturing polyacrylamide
35 gel. The results, presented in Fig. 2 show the superiority
of the invention compared to prior art. This superiority

could already be anticipated by comparing the shapes of the
HPLC chromatogram (A). The much broader peak obtained in the
standard synthesis reflects the presence of the shorter and
therefore more hydrophobe tritylated fragments. It is clear
5 from the scanned presentation of the gel separation (B) that
the product synthesized according to the present invention
is practically free of all truncated and depurinated
sequences. Moreover, this material contains substantially
lesser amount of n-1 fragments [(D) compared to (C)].
10

Example 9: Circularization of a padlock probe

A 91-mer oligonucleotide (M13C91: 5'-P-
15 GCCTGCAGGTCGACTCTAGA(T)$_{50}$CGGCCAGTGCCAAGCTTGCA-3')   were
synthesized according to the invention in order to test how
it would work as a padlock probe [Nilsson et al (1994),
supra] that is able to circularize in presence of an
oligonucleotide template (M135Ocomp: 5'-
20 TTTTTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTTTTT-3') and a
DNA-ligase. The ligation reaction was performed using 0.3
pmol of 5'-labelled probe and 5.5 pmol of template in a
volume of 10 μl 20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM
MgCl$_2$, 1 mM NAD, 0.01% Triton X-100 and 10 U of Tth DNA
25 ligase. The sample were subjected to one, two or three
cycles of 94°C for 15 seconds and 55°C for 10 min. The
reactions were cooled on ice and stopped by adding 10 μl of
loading buffer containing 50 % formamide and 10 mM EDTA
followed by a incubation at 65°C for 10 min. The reactions
30 were analyzed on a denaturing polyacrylamide gel as above.

The result of the ligation is shown in Fig. 3 which clearly
shows that all full-length oligonucleotides are ligatable as
well as most of the n-1 products, as would be expected if
35 the deletions were evenly distributed throughout the
sequence. It also shows the presence of many shorter

products that are ligatable and thus having intact 5'- and
3'- ends thereby revealing deletion events of more than one
nucleotide. Consequently, all nonligating shorter sequences
must contain deletions that prevent effective hybridization
5 to the targed DNA.

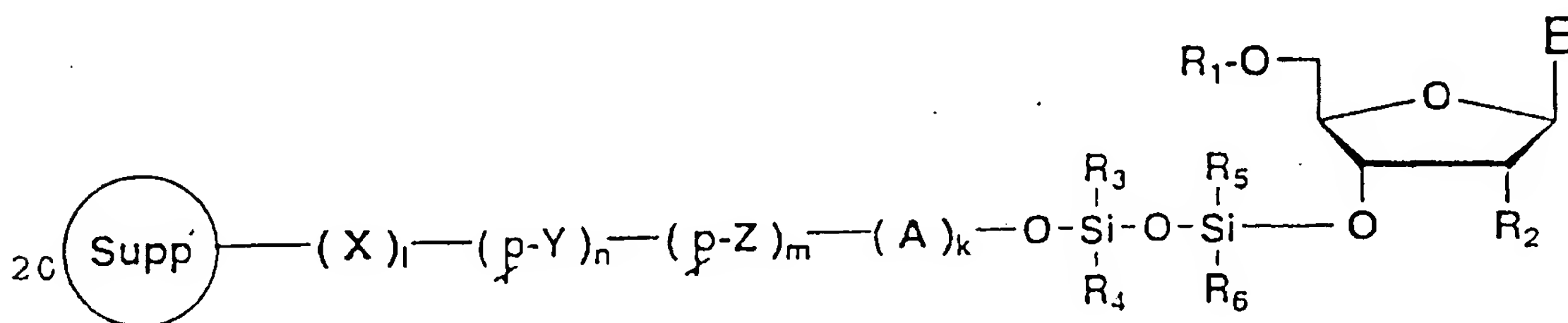
The present invention delivers products free of truncated
and post-depurination cleaved fragments, morover, the
10 isolated products contain much less n-1 fragments. The
amount of this n-1 material was estimated, in a study
performed on a short 5-mer sequence and using a standard
synthesis protocol ([Iyer et al, (1995), **14**(6) 1349-1357]),
to be roughly 3 to 5 %. By applying pre-cap procedure
15 according to Iyer et al and short contact time of ammonia
with the CPG support it was possible to lower this figure to
1.5 to 2 %. In contrast, in an experiment according to the
present invention for synthesis of a 20-mer using CPG
support (14), the amount of contaminating n-1 sequences was
20 found to be 1.9 %.

The support system of the present invention is not limited
to the solid-phase synthesis of oligonucleotides described
above. Another contemplated possibility is to use it as a
25 linker between support and hydroxyl containing components in
the combinatorial
chemistry ([Plunkett et al, (1995), J. Org. Chem., 60(19),
6006-6007]).

CLAIMS

1.   A support system, for solid phase synthesis of oligomers, comprising a support, a linker and a starting
5 compound of the oligomer, **characterized in** that the starting compound of the oligomer is bound to the support via a disiloxyl linkage.

2.   A support system according to claim 1, **characterized**
10 **in** that the starting compound is a nucleoside and the synthesis is an oligonucleotide synthesis.

3.   A support system according to claims 1 or 2, **characterized by** having the formula

15

wherein

B is a nucleoside or deoxynucleoside base; R_2 is -H, -OH, or
25 OR_7 in which R_7 is a protecting group; R_1 is a protecting group; R_3, R_4, R_5, R_6 taken separately each represent alkyl, aryl, cycloalkyl, alkenyl, aralkyl, cycloalkylalkyl, alkyloxy, aryloxy, cycloalkyloxy, alkenyloxy and aralkyloxy; Supp is a solid support; X is an anchoring group used for
30 covalent bonding to the support;

$(p-Y)_n$ and $(p-Z)_m$ are oligophosphotriester linkers, wherein p represents a phosphotriester, Y and Z are independently selected from a nucleoside and a rest of a diol, A is an aliphatic or aromatic group, n is a number between 0-50,
35 preferably 0-10, and k, l, m are each a numbers of 0 or 1, with the proviso that when m and n are 0 then l and k are 0

and with the proviso that when m > 0 then k is 0 and X is O or S.

4.   A support system according to claim 3, **characterized** in that the anchoring group is O, S or an amide function.

5.   A support system according to any one of the above claims, **characterized in** that R_1 is a trityl, monomethoxy trityl, dimethoxytrityl, pixyl or other higher alkoxy-substituted trityl- protecting groups, and B is adenine, guanine, cytosine, uracil, thymine, or inosine.

6.   A support system according to any one of the above claims, **characterized in** that R_3, R_4, R_5, R_6 are isopropyl.

7.   A method for synthesis of oligonucleotides on a solid support, **characterized** by the following steps:
(i) preparing a support system as defined in claim 3;
(ii) condensation of nucleotides onto the first nucleoside of the support system to synthesize an oligonucleotide;
(iii) removal of all protecting groups on the oligonucleotide exept the 5'-protecting group, and cleavage of apurinic sites formed during acid-catalysed deprotection;
(iv) cleavage of the full length product from the support; and
(v) purification of the oligonucleotide.

8.   A method according to claim 7, **characterized in** that in step (i) an oligophosphotriester linker $(p-Y)_n$ is synthesized on the solid support and that a starting nucleoside is bound to the linker via a $(p-z)_m$ linker and a disiloxyl group.

9.   A method according to claim 7, **characterized in** the disiloxyl linkage is cleaved selectively with tetra alkyl ammonium fluoride before step (v) and the purification is performed by reversed phase chromatography, using the 5'-protecting group as an affinity handle.

10.   A method according to claim 7, **characterized in** step
(v) is performed by exonuclease treatment whereby non-
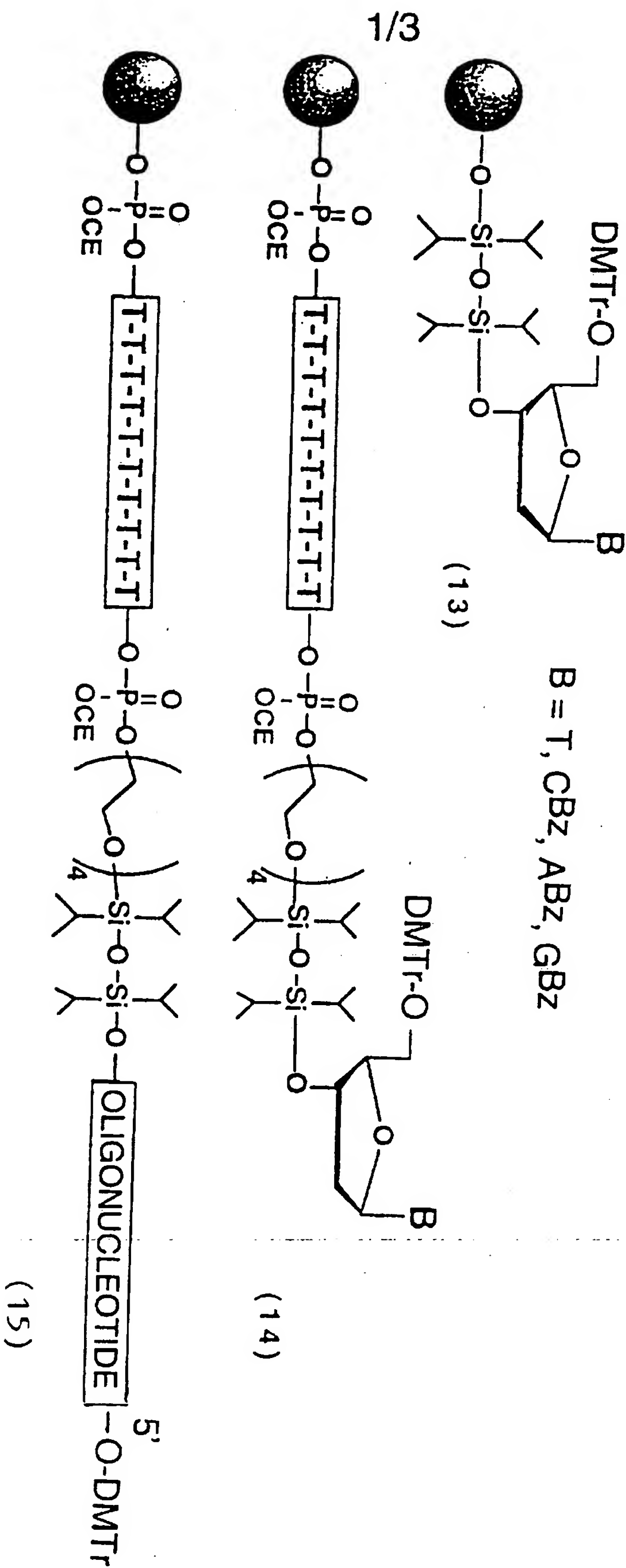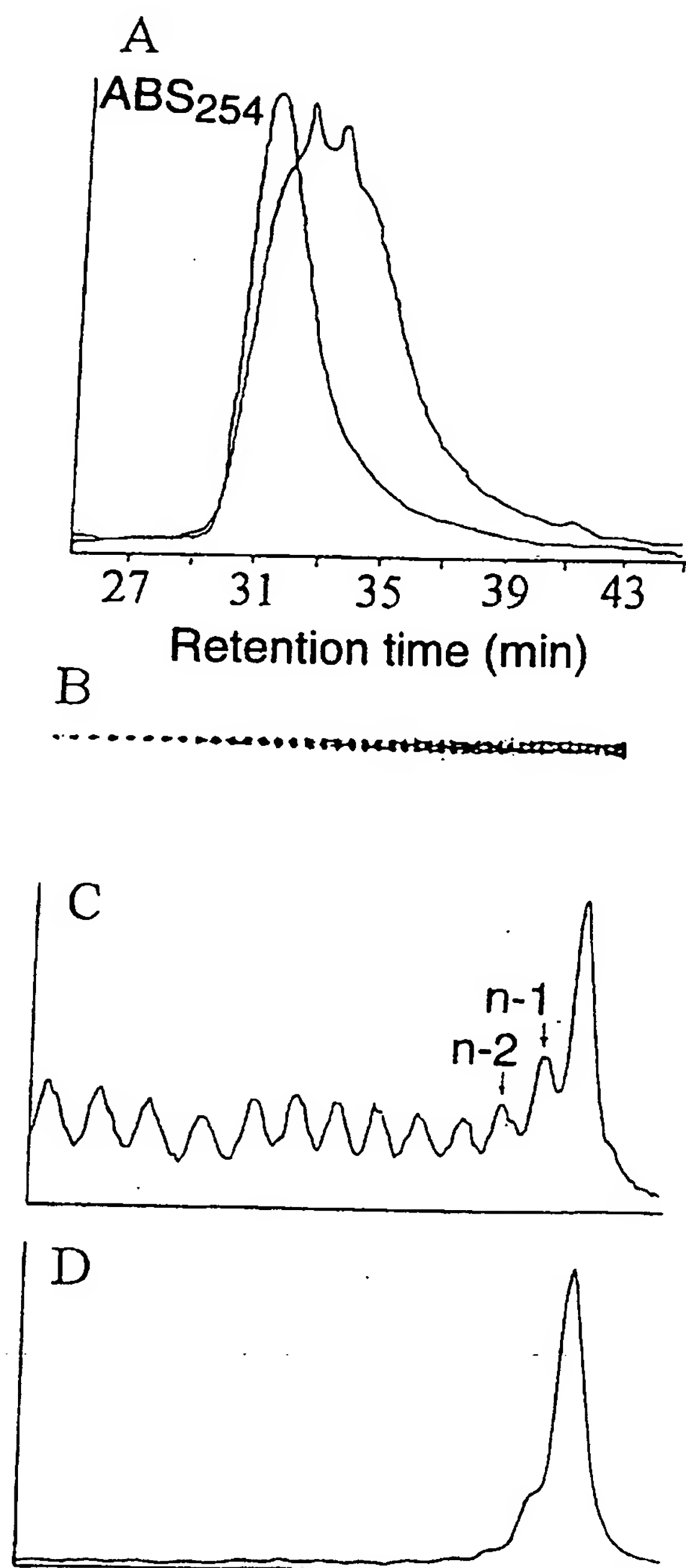protected oligonucleotides will be digested.
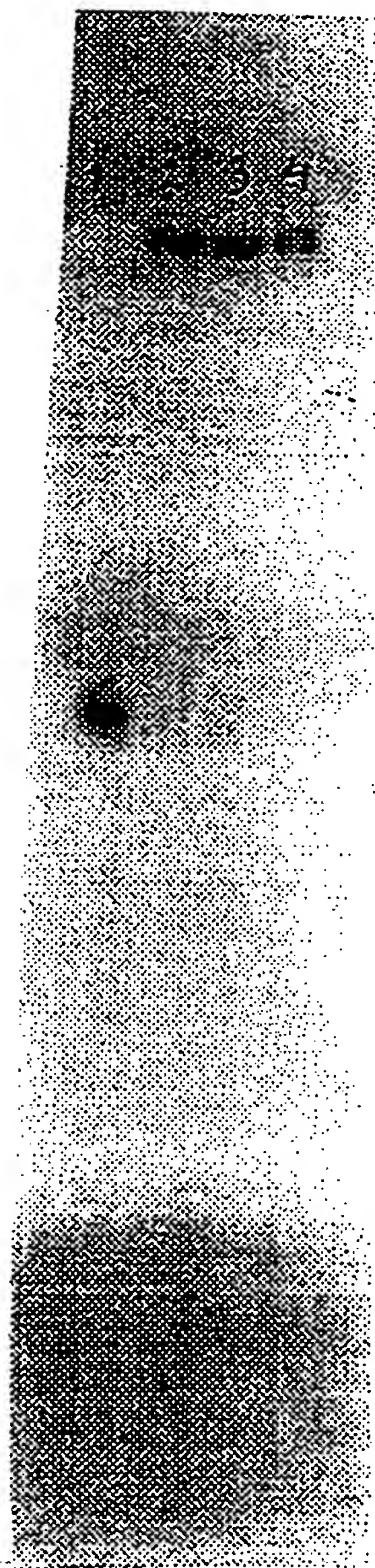
5

FIG. 1

FIG. 2

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01418

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07H 21/00, C07H 21/02, C07H 21/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9209615 A1 (PHARMACIA LKB BIOTECHNOLOGY AB), 11 June 1992 (11.06.92)	1-10
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A	WO 9313220 A1 (TEPNEL MEDICAL LIMITED), 8 July 1993 (08.07.93)	1-10
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A	WO 9204364 A1 (STERLING DRUG INC.), 19 March 1992 (19.03.92)	1-10
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☐ Further documents are listed in the continuation of Box C.   ☒ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 December 1997	08 -12- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Eva Johansson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

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